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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Clusterin is a protein that has been implicated in many normal physiological processes (tissue remodeling, sperm maturation) as well as many pathological processes (Alzheimer disease, atherosclerosis, cancer). Our laboratory became interested in clusterin when we identified it as an x-ray induced protein/transcript in human melanoma cells. Recently, we have identified two forms of this protein that are derived by alternative splicing of a single message. The secretory form of clusterin (sCLU) has been shown to have cytoprotective effects after cellular stress and injury. In contrast, a nuclear form of clusterin (nCLU) appears to be cytotoxic. Recently, Redondo *et. al* demonstrated that sCLU was overexpressed in breast cancer. sCLU over expression may provide a selective advantage in malignant cells. The most effective therapies for breast cancer after surgery include chemo- and radiation therapies. These therapies often fail as the tumor develop drug and radiation resistance. Our lab has shown that sCLU is induced by physiological doses of taxol, taxotere and radiation. Understanding the cellular and molecular responses of malignant and normal cells to these chemo- and radiation therapy would allow us to increase the efficacy of these treatments. Insight into the regulation of sCLU will allow us to better understand some of these processes.

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Introduction:

Clusterin (CLU) is a glycoprotein that has been implicated in a multitude of biological and pathological processes, including breast cancer(1). The function of clusterin is still unknown. Our laboratory identified CLU as a x-ray induced protein/transcript that could interact with the DNA double strand break repair protein, Ku70, implicating a possible role for CLU in DNA repair (2). This led us to propose the existence of a nuclear form of this protein (nCLU) (3). While secretory clusterin (sCLU) is thought to be cytoprotective, nCLU is cytotoxic(4,5).

The most effective therapies for breast cancer after surgery include chemo- and radio- therapies. These therapies often fail as the tumor develops drug and radiation resistance. Our lab has shown that sCLU is induced by physiological doses of taxol, taxotere and radiation (Criswell *et al.,* unpublished data). Understanding the cellular and molecular responses of malignant and normal cells to these therapies would allow us to increase the efficacy of these treatments. Insight into the regulation of sCLU will allow us to better understand these processes.

Determining the transcriptional regulation of sCLU will allow us to better understand its function after IR. As we began to investigate the regulation of sCLU, we noticed a correlation between sCLU expression and p53 status. p53 is a tumor suppressor protein that is found mutated in over 50% of all human cancers (6) and in 20% of all breast cancers (http://p53.curie.fr). The p53 protein is stabilized in response to genotoxic stress and acts as a transcription factor for genes resulting in either cell cycle arrest or apoptosis (7-9). Several lines of evidence suggest that sCLU is transcriptionally repressed by p53. (A) Wild-type p53 status in various breast cancer cell lines correlates with low basal levels of sCLU and, in general, no inducibility of sCLU after IR exposure. In contrast, breast cancer cell lines that contain mutant p53 or are null for p53 demonstrate high basal levels of sCLU; (B) HCT116 colon cancer cells that are p53 null show a dramatic induction of sCLU after IR as compared to cells that contain wild-type p53; and (C) MCF-7 cells that contain the HPV-16 E6 protein have an earlier induction of sCLU after IR as compared to cells without E6. Current work is focused on better

understanding the mechanisms underlying p53 suppression of the gene, as well as transcription factors needed for IR induction. We have included a new statement of work to cover these experiments.

Revised Statement of Work:

Aim 1: To investigate the transcriptional repression of secretory clusterin (sCLU) by the tumor suppressor protein, p53.

Task 1:

1. Screen various breast cancer cell lines for p53 and sCLU status before and after ionizing radiation (IR) exposure. This will allow us to examine sCLU basal levels and inducible levels after IR in breast cancer cells that contain either wild-type or mutant p53. We will use western blot analyses to examine sCLU protein levels.

Task 2:

1. Generate MCF-7 cells that stably express a clusterin promoter luciferase reporter construct that will allow us to monitor clusterin promoter activity in these cells before and after IR exposure.
    a. MCF-7 cells will be transfected with 1403 bp of the clusterin promoter that have been fused to a luciferase reporter (these cells will be referred to as MCF-7 1403 cells). Time course and dose response assays will be used to select a stable clone will be selected that mimics the behavior of the endogenous gene before and after IR exposure.
2. sCLU expression in MCF-7 cells that stably express the human papilloma virus E6 protein to abrogate p53 expression will be monitored via western blot and northern blot analyses. MCF-7 1403 cells will be stably transfected with the E6 protein and sCLU promoter activity will be monitored by luciferase assays.
3. p53 status will be further modulated in the MCF-7 1403 cells by stable expression of the dominant negative 273 mutant of p53. The effect of this mutant on sCLU expression will be

monitored by luciferase assays, western blot and northern blot analyses.

Task 3:

1. Isogenically matched HCT116 colon cancer cell lines that differ only in their p53 status will be used as a genetic model to investigate the effect of p53 on sCLU (wild-type p53 versus p53 null). We will switch to this genetic system in colon cancer cells since no equivalent system currently exists in a breast cancer model. Western and northern blot analyses will be used to determine sCLU expression in these cell lines.

2. sCLU expression will be monitored in mice that either contain wild-type p53 or are heterozygous/homozygous null for p53 status. These mice will be irradiated with 10 Gy or mock irradiated and major organs (heart, lung, spleen, colon, liver, kidney, brain, testes/ovaries) will be harvested 72 h later. These samples will be processed for protein and RNA for western and northern blot analyses respectively. Additionally, quantitative RT-PCR will be used to compare sCLU mRNA expression in the various tissues.

Aim 2: To determine the mechanism of p53 repression of sCLU transcription and to identify the transcription factors required for sCLU induction after IR exposure.

Task 1:

1. Deletion mutant analysis of the clusterin promoter luciferase reporter will be used to narrow down the region of the promoter required for CLU induction after IR. These constructs will be transiently transfected into HCT116 parental and p53 $^{-/-}$ cells and luciferase assays will be used to monitor promoter activity. These deletion mutants will allow us to define the site of the clusterin promoter that is required for p53 transcriptional repression as well as the transcription factors required for sCLU induction after IR exposure.

   a. Once the region required for sCLU induction is found, point mutations of the transcription factor binding sites within that specific region will be used to determine which transcription factors are required for induction.

   b. Electromobility shift assays (EMSA) will be performed to confirm the binding of the transcription factors to this site.

2. Chromatin immunoprecipitation assays (ChIP assays) will be used to demonstrate the physiological binding of these transcription factors after IR exposure and to determine whether p53 binds the clusterin promoter directly or causes the transcriptional repression indirectly.

Body of Grant Update:

Aim 1: To investigate the transcriptional repression of secretory clusterin (sCLU) by the tumor suppressor protein, p53.

Task 1: Screen various breast cancer cell lines for sCLU and p53 status.

Progress: We screened six breast cancer cell lines and looked for a correlation between p53 status and sCLU expression. In general, cells that contain wild-type p53 have low basal levels of sCLU protein as determined by western blot analyses. In contrast, cells with mutant p53 have high basal levels of sCLU protein (see Criswell *et al.*, JBC submitted 2002, in appendix).

Task 2: Examine sCLU expression in MCF-7 breast cancer cells in which the p53 status has been modulated.

1. Generate MCF-7 cells that stably express 1403 bp of the clusterin promoter fused to a luciferase reporter.

Progress: We have generated a stable cell line expressing the clusterin promoter luciferase reporter (MCF-7 1403 cells). Time course and dose response experiments were performed to show that these cells behaved similarly to the endogenous gene after ionizing radiation (IR) exposure (see Criswell *et al.*, JBC submitted 2002, in appendix).

2. Monitor sCLU expression in MCF-7 cells that stably express the HPV-16 E6 protein. The HPV-16 E6 protein binds p53 and targets it for rapid degradation leaving these cells essentially p53 null.

Progress: Western and northern blot analyses show that the MCF-7:E6 cells have higher basal levels of sCLU protein and message as compared to parental MCF-7 cells, suggesting that p53 is repressing transcription of this gene (see Criswell *et al.*, JBC submitted 2002, in appendix). We are currently in the process of developing

MCF-7 1403 cells that stably express the E6 protein as well as clones that contain a mutation in the E6 protein (K11E) that abrogates its ability to bind to p53.

3. p53 status will be modulated in the MCF-7 1403 cells by stable expression of the 273 dominant negative mutant of p53.

Progress: We are currently in the process of generating this cell line.

Task 3: Monitor sCLU status in the genetically matched HCT116 parental and $p53^{-/-}$ colon cancer cell lines with and without IR treatment.

1. Western and northern blot analyses show that HCT116 parental cells that contain wild-type p53 have low basal and inducible levels of sCLU. In contrast, the $p53^{-/-}$ cells show a dramatic increase of sCLU after IR exposure (see Criswell *et al.*, JBC submitted 2002, in appendix).

2. We are currently housing a $p53^{-/-}$ mouse colony obtained from Jackson Labs. In previous experiments, we have been able to isolate total RNA and polyA RNA from various tissues of untreated and irradiated mice. Unfortunately, there appears to be a very small quantity of sCLU message in these tissues, and we have not been successful in quantitating sCLU mRNA induction after whole body irradiation of these mice by northern blot analyses. In order to overcome this obstacle we are currently developing a technique that will allow us to use real-time quantitative PCR for this purpose.

Aim 2: To determine the mechanism of p53 repression of sCLU transcription and to identify the transcription factors required for sCLU induction after IR exposure.

Task 1: Deletion mutant analysis of the CLU promoter to determine regions of the promoter required for p53 repression of transcription and transcription factors required for sCLU induction after IR exposure.

1. Deletion mutants of the CLU promoter luciferase vector will be made to identify regions of the promoter involved in p53 transcriptional repression and involved in CLU induction after IR.

Progress: These deletion mutants have been made and preliminary data suggests that the transcription factor(s) Sp1 and/or $NF_{\kappa}B$ may be involved in the induction of sCLU after IR exposure (Fig. 1). The full length CLU promoter shows a 6-fold increase in activity after exposure to 10 Gy IR. Deletion of the 5' $NF_{\kappa}B$ and Sp1 sites

results in abrogation of promoter activation after IR. Further experiments will need to be done to confirm these results. Future experiments will include point mutations of the Sp1 and $NF_{\kappa}B$ DNA binding sites to confirm the importance of these sites in sCLU induction. Furthermore, electromobility shift assays (EMSA) will be used to show that Sp1 and $NF_{\kappa}B$ can bind to these sites within the promoter and chromatin immunoprecipitation assays will be done to demonstrate that Sp1 and $NF_{\kappa}B$ bind to the CLU promoter *in vivo*.

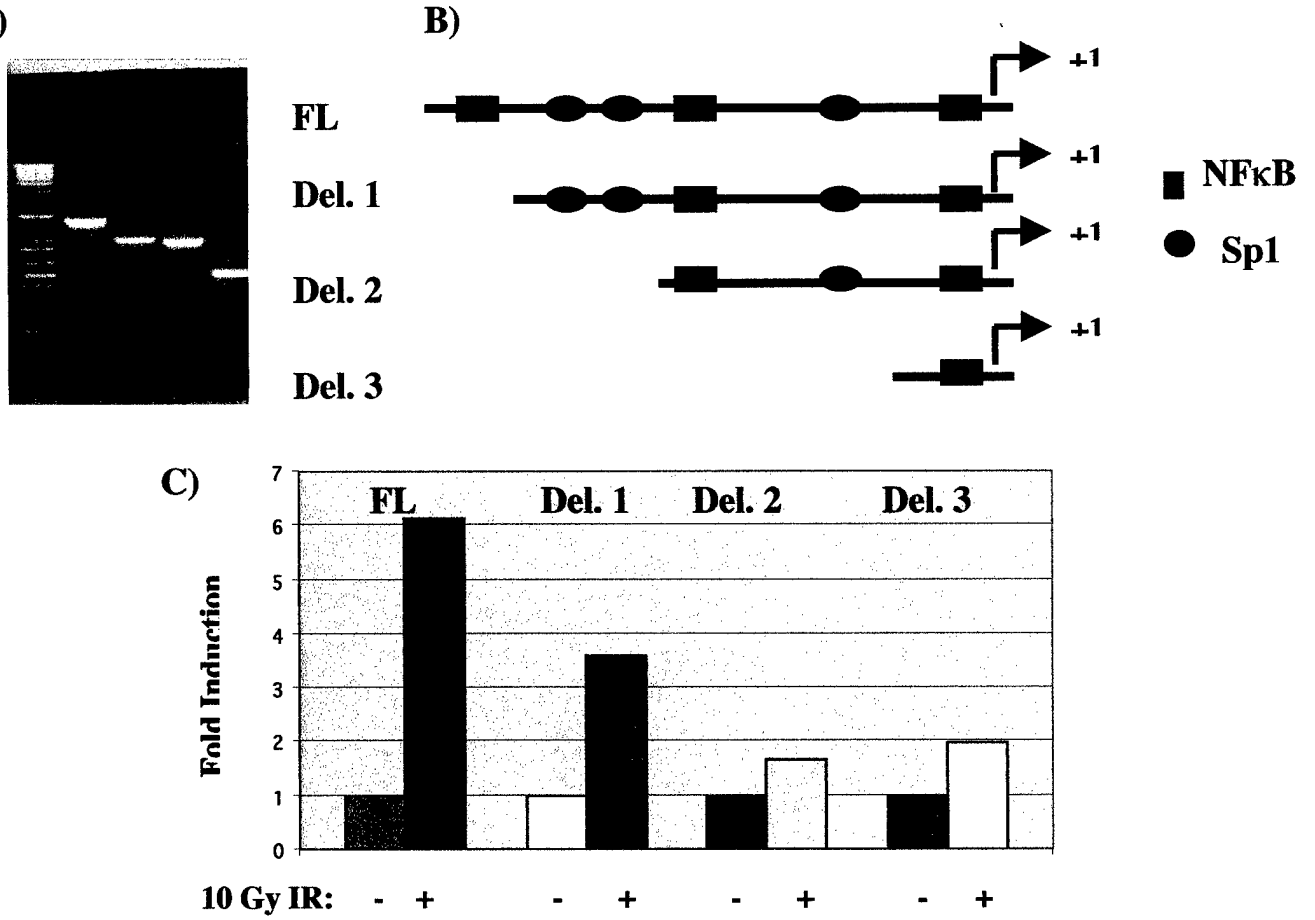

Figure 1: Preliminary data diagramming deletion mutants of the CLU promoter. (A) Agarose gel showing deletion mutants generated by PCR. (B) Schematic diagram illustrating location of each deletion within the CLU promoter (FL, full-length promoter; Del. 1, deletion 1; Del. 2, deletion 2; Del. 3, deletion 3). (C) Full length promoter and deletion mutants were transiently transfected into MCF-7 cells. Cells were irradiated 24 h after transfection and protein was harvested 48 h after irradiation for luciferase assays using the standard protocol from Promega.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of **key** research accomplishments emanating from this research.

We have accomplished the following objectives of this grant. We have determined/generated that:

Aim #1:

1. There is a correlation between p53 status and secretory clusterin (sCLU) status in various breast cancer cell lines.
2. A MCF-7 breast cancer cell line that stably expresses the clusterin (CLU) promoter luciferase reporter vector that behaves similarly to the endogenous sCLU gene.
3. MCF-7 cells that stably express the HPV-E6 gene have high basal levels of sCLU protein and message and little inducibility after IR.
4. HCT116 colon cancer cells that contain wild-type p53 show little inducibility of sCLU after IR, whereas HCT116 $p53^{-/-}$ cells show strong sCLU inducibility after IR.

Aim #2:

5. Deletion mutants of the CLU promoter have been generated.
6. Preliminary data suggests that Sp1 and $NF_{\kappa}B$ may be involved in sCLU induction after IR exposure.

REPORTABLE OUTCOMES:

CURRENT LIST OF PUBLICATIONS RESULTING FROM THIS AWARD
PAPERS PUBLISHED IN PEER-REVIEWED JOURNALS (Enclosed):

Criswell, T., Klokov, D., Lavik, JP., and Boothman, D.A. Transcriptional Repression of Clusterin by the p53 Tumor Suppressor Protein. 2002, submitted JBC.

ABSTRACTS AND PRESENTATIONS:

Poster Presentations:

1. Department of Energy/NASA Low Dose Radiation Workshop, Washington D.C., June 2001. Title: p53 repression of the secretory protein clusterin.
2. Low Dose Radiation Research Program Workshop, Rockville, MD, March 2002. Title: p53 repression of the secretory protein clusterin.
3. Radiation Research Society, Reno, NV, April 2002. Title: Transcriptional Repression of Clusterin by the p53 tumor Suppressor Protein.

Oral Presentations:

2. Radiation Research Society Young Investigator Presentation, Reno, NV, April 2002. Title: Transcriptional Repression of Clusterin by the p53 tumor Suppressor Protein.

DEVELOPMENT OF CELL LINES, TISSUE OR SERUM REPOSITORIES:

-MCF-7 breast cancer cells that stably express the CLU promoter luciferase reporter vector (MCF-7 1403 cells.

CONCLUSIONS:

The main progress on this grant has been the identification of p53 as a transcriptional repressor of sCLU. Studies have begun that will allow us to identify the mechanism of p53 repression as well as the transcription factors required for the induction of this gene after IR.

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Transcriptional Repression of Clusterin by the p53 Tumor Suppressor Protein

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Running Title: *Negative regulation of sCLU by p53*

Abbreviations: base pair, bp; CLU, clusterin; DSB, double strand break; IR, ionizing radiation; nCLU, nuclear clusterin; PC-4, Phthalocyanine-4; PDT, photodynamic therapy; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase pump; sCLU, secretory clusterin; TBP, TATAA binding protein; t-PA, tissue-type plasminogen activator; TGF-β, transforming growth factor-β; UV, ultraviolet radiation; xip8, x-ray induced protein-8

SUMMARY

Recent data indicate that the clusterin protein has both cytoprotective as well as cytotoxic activities. Our previous data strongly suggested that the secretory form of the clusterin protein was cytoprotective and induced after very low, nontoxic doses of ionizing radiation (IR: $\geq$0.02 Gy). In contrast, a nuclear form of the clusterin protein was induced by higher doses of IR (>1 Gy) and elicited cytotoxic apoptotic responses. Thus, clusterin appears to represent a molecular switch between cell death and survival.

We investigated the regulation of secretory clusterin after ionizing radiation and examined the potential regulation of secretory clusterin mRNA production and protein synthesis by the tumor suppressor protein, p53. IR stimulated clusterin promoter activity, with concomitant increases in mRNA and protein, in log-phase MCF-7 cells 24-72 h post-IR. Expression of human papillomavirus E6 protein in MCF-7 cells enhanced basal clusterin protein and mRNA levels, and augmented clusterin synthesis after IR. Isogenically matched HCT116 colon cancer cell lines that differ only in p53 or p21 status, confirmed a role for p53 in the transcriptional repression of secretory clusterin. Repression of clusterin by p53 may be important for the cascade of events leading to cell death.

Keywords: clusterin, p53, ionizing radiation

INTRODUCTION

Secretory clusterin (sCLU) is a sulfated glycoprotein that was implicated in many physiological and pathological processes, including tissue remodeling (1), complement inhibition (2,3), lipid transport (4,5), multiple sclerosis (6), atherosclerosis (7,8), and Alzheimer's disease (9-11). Elevated levels of sCLU protein and mRNA were noted in several different types of human malignancies (12,13), and forced over-expression of sCLU in transformed cell lines resulted in an increased resistance to various chemotherapeutic agents (14). In addition, abrogation of CLU mRNA expression following antisense expression lead to modest chemo-sensitization in various cell lines (15-18).

Our laboratory identified CLU as a x-ray inducible protein/transcript (xip8) (19) that associated with the DNA double strand break (DSB) repair protein, Ku70. We demonstrated that a nuclear form of the clusterin protein (nCLU) bound Ku70 (20,21), inhibited Ku70/Ku80 end binding activity (21), and caused cell death when over-expressed in tumor cell lines (20), suggesting a potential role for this protein in DNA repair as well as cell death responses after IR. However, during these studies we also found that sCLU did not associate with Ku70 and was induced by much lower, nontoxic doses of IR. In fact, sCLU was induced at ~0.02 Gy, a dose that was found to be growth-stimulatory and cytoprotective in many human cancer cells (22).

Although the regulation of sCLU following estrogen and testosterone exposures have been investigated (23,24), the regulatory control of sCLU synthesis after IR has not been elucidated. sCLU mRNA and protein synthesis in human cells is induced after various cytotoxic stresses, including treatment by many anti-tumor agents. However, unlike the pathways involved in sCLU transcriptional regulation after hormonal regulation, the mechanisms of sCLU induction at the promoter, transcript and protein levels after cytotoxic stress (including after IR), have not

been elucidated. This investigation represents a beginning of such studies, whose completion could lead to the elucidation of signaling events for improved radiotherapy against cancer.

The p53 tumor suppressor gene is mutated in over half of all human tumors (25), which commonly leads to a stable protein with loss of function. Wild-type p53 protein is stabilized after cellular stress and acts as a transcription factor for various downstream genes, including Bax, p21 and GADD45, resulting in either cell cycle arrest or apoptosis (26-28). p53 has a short half-life and is normally maintained at low basal levels within the cell. Cellular stresses, such as ultraviolet radiation (UV), IR, hypoxia, oxidative stress, inhibition of microtubules and nucleotide depletion result in the rapid stabilization and accumulation of the p53 protein by phosphorylation and/or acetylation (29). Post-translational modification of p53 was proposed to be regulated by Chk2, DNA-PK, ATM, ATR and p300/PCAF. Stabilization of p53 may then result in a G_1 cell cycle checkpoint arrest via induction of p21 (30,31) and/or cell death through the induction of apoptotic proteins, such as Bax (32,33). After stress-induced stabilization, p53 forms homotetramers that bind to two copies of a ten base pair (bp) nucleotide sequence (5'-PuPuPuC(A/T)(T/A)GpyPyPy-3') divided by a 0-13 bp spacer.

Our knowledge concerning p53-repressed genes is less than that of genes that are transactivated by p53. Examples of p53-repressed genes include presenillin 1 (34), hsp70 (35), cyclins A (36) and B (37), Map-4 (38) and cdc2 (39). The overall significance of genes repressed by p53 is unknown.

In this study we investigated the regulation of sCLU in various human tumor cells after IR exposure. We showed that sCLU is up-regulated at the message level in a very delayed fashion after IR treatment. IR-induction studies of CLU promoter activity, CLU mRNA accumulation, and sCLU protein synthesis confirmed that sCLU expression occurred in a

delayed fashion (24-72 h) post-irradiation. Furthermore, the low levels of IR (>0.02 Gy) required to induce sCLU and the dramatic accumulation of sCLU protein following taxol, PMA or thapsigargin (a sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump inhibitor that causes dramatic alterations in intracellular calcium homeostasis) exposures, strongly suggest that DNA damage is not required for the activation of this gene in MCF-7 breast cancer cells. Forced expression of the papillomavirus E6 protein, as well as isogenically matched cell lines that differ only in their p53 status, were used to demonstrate a role for p53 in the transcriptional repression of sCLU in unirradiated cells. Our data strongly suggest that the CLU promoter, as well as sCLU mRNA production and protein synthesis are repressed by the tumor suppressor protein, p53.

EXPERIMENTAL PROCEDURES

Cell Culture- MCF-7:WS8 human breast cancer cells (MCF-7) were obtained from Dr. V. Craig Jordan (Northwestern University; Evanston, IL). MCF-7 cells were transduced by retroviral transfer with a CMV-driven papillomavirus E6 vector by Dr. Jordan's lab, and subsequently subcloned into cell lines with varying E6 expression. The E6-D MCF-7 cell line showed no p53 expression, even after IR exposure. Human colorectal carcinoma HCT116 parental, $p53^{-/-}$, and $p21^{-/-}$ cell lines were developed (40) and generously provided by Dr. Bert Vogelstein (Johns Hopkins University; Baltimore, MD). These cell lines were confirmed by our laboratory to be null for p53 and p21 respectively by western blot analyses. MCF-7, ZR-75-1, T47-D, BT474, MDA-MB-231 and MDA-MB-468 cell lines were grown in RPMI 1640 cell culture media supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified incubator with a 5% CO_2-95% air atmosphere as described (41). MCF-7:E6D cells were maintained in 0.4 mg/ml geneticin (Life Technologies; Carlsbad, CA). All HCT116 cells were grown in DMEM

cell culture media supplemented with 10% FBS at 37°C in a humidified incubator with a 10 % CO_2-90% air atmosphere. All experiments were initiated by spreading 5 x 10^5 log-phase growing cells per 10-cm^2 tissue culture dish in the appropriate medium in the absence of any antibiotics (e.g., geneticin). All cell lines were free from mycoplasma contamination.

IR Treatments- Cells were irradiated as previously described (19). Briefly, cells were irradiated with ^{137}Cs gamma rays at a dose rate of 0.87-0.92 Gy/min, using a Shepard Mark Irradiator. Untreated cells were mock-irradiated as described (19).

Northern Blot Analyses- Total RNA was extracted from control or irradiated MCF-7 or HCT116 cells using Trizol (Life Technologies; Carlsbad, CA) as per the manufacturer's instructions. Ten - twenty micrograms (10 - 20 μg) total RNA was separated on a denaturing formaldehyde gel, transferred to a Hybond membrane (Amersham Pharmacia; Sunnyvale, CA) and probed with ^{32}P-labeled full length CLU or 36B4 as a loading control as previously described (42). Signal was quantitated using ImageQuant software version 4.1 (Molecular Dynamics; Sunnyvale, CA) on a Molecular Dynamics phosphoimager. CLU mRNA levels were normalized to the untreated control and to 36B4 mRNA levels for fold induction calculations as previously described (42); 36B4 mRNA levels do not vary with stress or cell cycle position (42).

Luciferase Assays- All luciferase assays were performed using the Luciferase Assay System (Promega; Madison, WI). MCF-7 cells were stably transfected with 1403 bp of the human clusterin promoter in a luciferase reporter plasmid using a standard liposome protocol (Effectene, Qiagen, Valencia, CA). The plasmid was a generous gift from Dr. Martin Tenniswood (University of Notre Dame; Notre Dame, IN). These cells (MCF-7 1403 cells) were seeded in 6-well plates at approximately 50% confluency. Cells were irradiated at the indicated dose and harvested at various time points in 1X reporter lysis buffer (Promega; Madison, WI).

Each dose/time point was completed in triplicate and a Student's T-Test was performed to determine statistical significance.

Western Blot Analyses- Whole cell extracts from control or irradiated cells were extracted in RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0) and separated on a 10% gel by SDS-PAGE western blot analyses as previously described (20). Proteins were transferred to Immobilon-P (Millipore; Bedford, PA) and probed with the B-5 human sCLU monoclonal antibody, the DO-1 human p53 monoclonal antibody and the human Ku70 polyclonal antibody. All antibodies were obtained from Santa Cruz and used as per manufacturer's instructions. Ku70 was used as a control for equal loading of protein, since its levels remain unaltered after IR under the time-frame of our experiments.

Cell Cycle Analyses- HCT116 parental, $p53^{-/-}$ or $p21^{-/-}$ cells were synchronized by allowing them to grow to 100% confluence on 10-cm^2 tissue culture dishes as described (19,43). Cells were then maintained for 48 h in serum-free medium to maximize G_0-G_1 arrest. Cells were released from the dual confluence and serum-free cell cycle arrest by trypsinization (using 0.05% trypsyn with 0.53 mM EDTA) and replated at 1:8-1:10 dilution in DMEM containing 10% FBS under conditions described above. For irradiation experiments, cells were irradiated 10 h after release from the cell cycle arrest and prior to the 16 h p53 checkpoint (44). Concurrent flow cytometric and western blot analyses were performed as follows. For flow cytometric analyses, at the indicated time points after release, cells were dissociated by scraping into 1X PBS, collected by centrifugation (500 x g), fixed in 90% ethanol, and stored at –20 ^{0}C until analyzed. Cells were washed two- to three-times in ice-cold PBS containing 1% FBS, and stained with 33 mg/ml propidium iodide (Sigma; St. Louis, MO), 1.0 mg/ml RNase A (Sigma),

and 0.2% NP-40 (Calbiochem; La Jolla, CA) at 4 ^{0}C overnight. Stained nuclei were then analyzed for DNA content by propidium iodide fluorescence using Coulter Epics XL (Beckman Coulter Electronics; Miami, Fl). Data was analyzed using ModFit LT, version 2.0 software (Verify Software House; Topsham, ME). Western blot analyses completed simultaneously with the flow cytometric analyses described above were performed as described under 'western blot analyses'. Flow cytometric and western blot results shown are representative of experiments performed at least three times.

RESULTS

sCLU is transcriptionally upregulated after ionizing radiation. Our laboratory previously showed that CLU was a x-ray-induced protein (xip8) (42), and that the nuclear form of this protein (nCLU) played a cytotoxic role in the cell (20). In contrast, sCLU was shown by other laboratories to be cytoprotective (45,46). To further characterize the induction of sCLU after IR exposure, northern blots were used to determine if sCLU induction in log-phase MCF-7 human breast cancer cells after IR exposure was due to an increase in sCLU mRNA or protein stabilization (Fig. 1A). MCF-7 cells were exposed to 10 Gy and total RNA was isolated at various post-treatment times (4, 24, 48, 72 and 96 h). Total RNA (10 μg) was used for northern blot analyses. Blots were probed with [^{32}P]-labeled full-length CLU and 36B4 cDNA to control for loading (42), as described in 'Experimental Procedures'. As expected, induction of CLU mRNA mirrored induction of protein (20), with accumulations of each occurring at 24 h post-irradiation. Maximal induction of sCLU mRNA and protein (7- to 10-fold) over untreated cells

occurred 72 to 96 h after 10 Gy. Induction of sCLU at the message level was confirmed using RNase protection assays (data not shown).

We then performed IR dose-response experiments in log-phase growing MCF-7 cells, examining CLU protein and mRNA accumulations 72 h after exposure. MCF-7 cells were treated with increasing doses of IR (from 0.02 Gy up to 10 Gy). RNA was isolated 72 h after exposure and 10 μg of total RNA was used for northern blot analysis. As previously reported for sCLU protein induction at 72 h post-IR (20), sCLU mRNA was induced 2-fold at 2 cGy with a maximal induction of 22 fold at 5 Gy (Fig. 1B). Interestingly, we observed a drop in CLU mRNA induction following 10 Gy in log-phase MCF-7 cells, possibly a result of cell death at this later time post-IR.

To determine if IR-induced CLU transcription increases were due to production of new message or to a decrease in mRNA degradation, we examined CLU promoter activity. For these experiments, we generated a stable MCF-7 cell line containing 1403 bp of the human CLU promoter with a downstream luciferase reporter gene as described in 'Experimental Procedures'. Transient transfections with the CLU reporter plasmid were not possible, since all transfection methods examined induced the CLU promoter in MCF-7 cells, as well as endogenous sCLU gene/protein expression (data not shown); induction of sCLU may be triggered by cell membrane insult (47). Dose-response (Fig. 1C) and time-course (Fig. 1D) assays of exogenous CLU promoter activation in MCF-7 1403 cells were performed to show that this clone behaved similarly to the endogenous CLU gene before and after IR exposure. The CLU promoter was activated in a time- and dose-dependent manner similar to that previously shown for sCLU protein and mRNA (Fig. 1A & B), although induction was not observed at lower doses of IR (below 1.0 Gy) as was seen by northern blot analyses.

sCLU is a stress protein induced by a variety of cytotoxic agents. The idea that sCLU is a general stress-induced cytoprotective protein prompted us to investigate whether various cytotoxic agents could also induce expression of sCLU protein. Table 1 lists agents that induced sCLU in MCF-7 cells. These agents included ultraviolet radiation (UV), topoisomerase I and IIα poisons, microtubule stabilizers/destabilizers and various other agents (e.g. PMA, thapsigargan) that do not cause direct damage to DNA. The only agent tested that did not induce sCLU expression was β-lapachone, a novel apoptotic drug that quickly depletes cellular ATP in NQO1-expressing MCF-7 cells (48), which may result in inhibition of new protein synthesis. These data suggest that damage to DNA may not be required for sCLU induction, and that alterations in calcium homeostasis (indicated by thapsigargin induction of sCLU, Table 1) may play a common triggering role in the induction of this gene.

Influence of p53 status on basal and inducible levels of sCLU. Since p53 is a transcription factor that is activated by most of the agents in Table 2, we examined the relationship between p53 status and sCLU expression. Various human breast and colon cancer cell lines with known mutations in p53 were examined for basal and IR-inducible sCLU levels as monitored by western blot analyses and described in 'Experimental Procedures'. With one exception, cells expressing mutant p53 exhibited increased basal levels of sCLU (Table 2). This was apparent in T47-D, BT-474 and MDA-MB-468 cells. The one exception was mutant p53-expressing MDA-MB-231 cells. These cells appear to lack basal or IR-inducible protein expression, although low levels of CLU mRNA were detected by RT-PCR (Table 2). In contrast, cells expressing wild-type p53 (MCF-7 parental, ZR-75-1, HCT116 parental, HCT116 $p21^{-/-}$) expressed low or no detectable basal levels of sCLU (Table 2).

Consistent with a possible negative regulation of sCLU by endogenous wild-type p53, MCF-7 cells infected with a retrovirus expressing the human papillomavirus E6 protein exhibited high basal levels of sCLU with a low level IR-inducibility of the protein (Table 2). Additionally, HCT116 $p53^{-/-}$ cells containing a somatic deletion of p53 showed strong induction of sCLU after IR exposure compared to parental HCT116 or HCT116 $p21^{-/-}$ cells; HCT116 $p21^{-/-}$ cells were somatically knocked out for the p21 gene in a similar manner as $p53^{-/-}$ HCT116 cells (40).

Apparent suppression of sCLU expression by wild-type p53 did, however, appear to be overcome by as yet unknown factors important for IR-inducibility. For example, MCF-7 cells demonstrated a high capacity to induce sCLU after IR, whereas wild-type p53 expressing ZR-75-1 cells did not induce measurable increases of sCLU, and HCT116 $p21^{-/-}$ and HCT116 parental cells only slightly induced sCLU levels. This difference may be due to differential expression of as yet undescribed transcription factors or signal transduction mechanisms required for sCLU induction; ZR-75-1 cells may lack certain factors required for sCLU induction. In general, these results led us to propose that sCLU was a novel p53 repressed gene.

MCF-7 cells expressing the HPV-16 E6 protein have high basal levels of sCLU and show little inducibility after IR exposure. To further elucidate the effect of p53 on sCLU expression, we compared parental MCF-7 cells to isogenically matched cells stably transfected with the E6 protein, as described in 'Experimental Procedures'. Western blot analyses were used to compare induction of sCLU in these cells after 10 Gy IR (Fig. 2A). Ku70 was used to control for equal loading. sCLU was induced in parental MCF-7 cells starting at 24 h and peaking at 72 h post-IR treatment. The 40 kDa secretory form of the protein appears as a smear by western blot analyses due to variations in glycosylation of the protein. The position of sCLU protein was

confirmed by separating whole cell protein extracts under non-reducing SDS-PAGE conditions (data not shown), in which sCLU protein appears as an 80 kDa α/β heterodimer (20). sCLU basal levels were higher in control mock-irradiated MCF-7:E6 cells compared to parental MCF-7 cells. As expected, p53 protein was not detected before or at various times after 10 Gy in the MCF-7:E6 cells. We also noted that sCLU induction over basal levels after 10 Gy IR was less in MCF-7:E6D cells compared to the parental MCF-7 cells, presumably due to the high basal levels already present in these cells. Finally, in MCF-7 clones expressing suboptimal E6 levels, where reduced p53 levels were observed (as in MCF-7:E6A cells), western blot analyses revealed an intermediate phenotype between MCF-7 parental and MCF-7:E6D cells. p53 levels were only present after IR damage, and sCLU levels were induced with the same kinetics from a slightly elevated basal level (data not shown).

Northern blot analyses demonstrated that sCLU mRNA was also higher in non-irradiated mock-treated MCF-7:E6 cells compared to the parental MCF-7 cells (Fig. 2B). As previously shown (Fig. 1), sCLU mRNA in MCF-7 parental cells was induced after 10 Gy IR, with accumulation noted at 24 h post-IR exposure and a maximal induction (7-10 fold over basal levels) occurring 72-96 h post-treatment. Mock IR-treated MCF-7:E6 cells expressed 3-fold higher basal sCLU mRNA levels compared to mock IR-treated parental MCF-7 cells. Although similar IR-induction kinetics of sCLU transcript levels were observed in MCF-7:E6 cells as in IR-treated MCF-7 parental cells, only an ~2-fold accumulation of mRNA was noted, consistent with sCLU protein expression. Thus, loss of basal p53 expression by exogenous E6 expression resulted in an increase in basal sCLU steady state transcript and protein levels, with reduced overall IR-induction responses.

Human HCT116 colon cancer cells with a somatic p53 deficiency showed greater IR-inducible sCLU levels. To confirm the repression of sCLU by p53 we used isogenically matched human HCT116 colon cancer cell lines that differed only in their p53 or p21 status. Western blot analyses showed that IR-treated parental HCT116 cells stabilized and accumulated p53 (i.e., expressed wild-type p53), but increases in steady state levels of sCLU were not detected (Fig. 3A); in other blots, sCLU could be detected with no more than a 2-fold increase in sCLU levels at various times post-treatment (up to 130 h). In HCT116 cells, we were not able to observe intracellular mature sCLU levels (the reduced 40 kDa secretory protein) and IR-induced sCLU induction responses were monitored via the ~60 kDa sCLU precursor protein (psCLU). In contrast to parental HCT116 cells, HCT116 $p53^{-/-}$ cells dramatically induced sCLU after 10 Gy IR. $p53^{-/-}$ HCT116 cells also mimicked MCF-7:E6D cells in that the basal level of sCLU was elevated compared to parental HCT116 cells. Northern blot analyses confirmed induction (2- to 3-fold) of steady state sCLU mRNA in HCT116 $p53^{-/-}$ cells (Fig. 3B), whereas, $p53^{+/+}$ parental HCT116 cells showed little or no induction of sCLU mRNA after 10 Gy IR; post-treatment times of up to 96 h were examined in IR-treated HCT116 parental and $p53^{-/-}$ cells. Additionally, sCLU protein was induced in the HCT116 $p53^{-/-}$ by 150 ng/ml nocodazole, 50 nM taxol and 50 nM topotecan (data not shown). These agents did not induce sCLU in the wild-type p53 expressing parental cells.

HCT116 $p21^{-/-}$ cells do not induce sCLU after IR exposure. To demonstrate that induction of sCLU in p53 null cells was specific for p53, we utilized HCT116 cells that were somatically knocked out for the p21 gene (40). As in HCT116 parental cells, sCLU protein was not induced in HCT116 $p21^{-/-}$ cells after 10 Gy compared to mock-treated cells, as determined by western blot analyses (Fig. 4A). Furthermore, steady state sCLU mRNA levels were also not

induced in $p21^{-/-}$ HCT116 cells after 10 Gy (Fig. 4B). Analyses of p53 and p21 protein accumulation after IR revealed that HCT116 $p21^{-/-}$ cells stabilized p53 with similar kinetics as HCT116 parental cells, but did not show p21 induction responses (data not shown) after any treatment for up to 96 h.

sCLU is not cell cycle regulated. An alternative explanation for sCLU induction and subsequent repression by p53 could be that sCLU is regulated by the cell cycle. Recent reports suggested that sCLU may be expressed exclusively in quiescent cells (49). Thus, we would expect that in serum starved and confluence-arrested HCT116 cell lines, sCLU expression would decrease as cells progress through G_1 and S phases of the cell cycle. To address this issue, HCT116 parental, $p53^{-/-}$ and $p21^{-/-}$ cells were arrested in the G_0/G_1 phase of the cell cycle by dual serum-starvation and confluence-arrest conditions as described (50) and explained in 'Experimental Procedures'. Synchronized HCT116 parental, $p53^{-/-}$ or $p21^{-/-}$ cells were mock irradiated (Figs. 5A, C and E) or irradiated (10 Gy, Figs. 5B, D and F) at 10 h post-release (a time prior to the p53 cell cycle G_1 checkpoint) and cell cycle checkpoint alterations and sCLU expression were monitored. At 10 h post-release, cells with wild-type p53 and a functional G_1 arrest checkpoint (Fig. 5A and B) arrested in G_1 as described (44). Irradiated HCT116 parental cells subsequently entered S-phase 14-16 h after release, with concomitant decreases in G_0/G_1 cells. As expected, HCT116 parental cells containing wild-type p53 exhibited a prolonged G_1 arrest after IR. For example, 18 h after release (8 h after 10 Gy IR exposure), more than 45% of HCT116 parental cells were still arrested in G_1.

In contrast, HCT116 cells with somatic deletions of p53 (Fig. 5C and D) or p21 (Fig. 5E and F), entered S-phase earlier, with accumulation of S-phase cell populations occuring at 12-16

h, accompanied by concomitant decreases in G_0/G_1 cells. At 18 h after release (8 h after 10 Gy IR exposure), only 14% and 28% of $p53^{-/-}$ and $p21^{-/-}$ cells, respectively, remained in G_1, while 80% and 66% of cells, respectively, proceeded into S phase. As expected, IR-treated HCT116 $p53^{+/+}$ cells arrested in G_1 and exhibited delayed S or G_2/M phase entry compared to either $p53^{-/-}$ or $p21^{-/-}$ HCT116 cells.

Western blot analyses of non-irradiated synchronized HCT116 parental, $p53^{-/-}$ and $p21^{-/-}$ cell populations indicated that the levels of sCLU did not change relative to basal levels throughout the cell cycle. Interestingly, sCLU was dramatically induced only in IR-exposed synchronized HCT116 $p53^{-/-}$ cells with similar induction kinetics (maximal accumulation observed between 24-72 h) as found in IR-treated asynchronous log-phase HCT116 $p53^{-/-}$ cells (Fig. 3). In contrast, only slight or no sCLU induction responses were noted in synchronized HCT116 parental or $p21^{-/-}$ cells. Therefore, these data strongly suggested that the ability of p53 to repress endogenous sCLU gene expression was independent of cell cycle checkpoint arrest responses.

DISCUSSION

Our data strongly suggest that p53 suppresses expression of sCLU. A limited screen of breast cancer cell lines indicated an inverse relationship between p53 status and sCLU expression. We subsequently used two cell line model systems to investigate the role of p53 in the transcriptional regulation of sCLU. We compared induction of sCLU after IR exposure in MCF-7 breast cancer cells that express wild-type p53 to MCF-7 cells that were transfected with and express the HPV-16 E6 protein. The E6 protein binds to p53 and targets it for rapid degradation through the proteasome pathway, leaving these cells essentially p53 null (51). We

found that the basal level of sCLU was high in unirradiated MCF-7 cells that express the E6 protein, and that the levels of the protein were only slightly induced after IR exposure (Fig. 2). Furthermore, sCLU basal levels were low in MCF-7 cells and intermediate in MCF-7 cells that expressed low levels of E6 that were not sufficient to prevent p53 induction after IR (data not shown).

We also examined the induction of sCLU after IR exposure in isogenically matched HCT116 colorectal cell lines that differed only in their p53 or p21 status (Fig. 3). Low basal levels and no induction of sCLU after IR were observed in parental HCT116 cells that contained wild-type p53. In contrast, p53 null cells demonstrated strong induction of this protein after physiological doses of IR exposure. Furthermore, we noted a correlation between sCLU protein levels and mutant p53 status (Table 2) in human breast cancer cells, wherein apparent loss of p53 function via mutation appears to result in elevated sCLU levels. In addition, we noted a similar relationship between p53 status and sCLU expression in prostate cancer cell lines, where $p53^{+/+}$ LNCaP cells have very low basal sCLU expression and p53-mutant DU145 or p53-null PC3 cells express high levels of sCLU (data not shown). Thus, the relationship between p53 status and sCLU expression and induction after IR appears to be a general phenotype and not unique to specific cell lines.

The effect of IR exposure on sCLU expression in MCF-7 cells was different than that found in HCT116 parental cells, even though both cell lines express wild-type p53. HCT116 parental cells did not induce sCLU after IR exposure, and on some blots, sCLU was not detected at all. In fact, MCF-7 cells appear to be the only wild-type p53-expressing cell line examined to date that strongly induced sCLU. It may be that MCF-7 cells overexpress the transcription factors needed for induction of this protein, while HCT116 cells maintain lower levels, which are

in turn suppressed by wild-type p53 even after IR exposures (see model, Fig. 6). It appears that these transcription factors may be constitutively expressed in MCF-7 cells, since E6 expression greatly enhanced sCLU expression in MCF-7 cells without IR exposure. It should be noted that expression of E6 in MCF-7 cells (i.e., MCF-7:E6D cells) lost its ability over time to abrogate p53 expression as these cells were cultured; as basal p53 expression was noted even with E6 expression, sCLU basal levels decreased. The factors needed for sCLU induction have not been elucidated. Previously, we showed that SP1 and NF-κB can bind to the tissue-type plasminogen activator (t-PA) promoter (52), another IR-inducible transcript (xip), and that this binding corresponded to the induction of the t-PA promoter (52). As with all of the known xip promoters, the CLU promoter does contain SP1 and NF-κB binding sites and these sites may be involved in sCLU induction after IR exposure. Furthermore, MCF-7 cells appear to have constitutive NFκB expression (Miyamoto *et al.*, personal communication), which could be one factor in the expression of sCLU in these cells before and after IR. Alternatively, Jin *et al.* demonstrated that sCLU protein and message could be induced by transforming growth factor β (TGF-β1) via a modulation of c-fos (53), and recent reports have documented TGF-β1 induction after IR (54). Such a mechanism may explain the delayed expression of sCLU after IR. It was suggested by Cervellera *et al.* (55) that B-myb may transactivate sCLU expression through a B-myb binding site. This report also suggested that increased sCLU protein expression, due to B-myb transactivation, resulted in an increase in survival in neuroblastoma cells after doxorubicin treatment. Unfortunately, analyses of the transcription factors and DNA elements within the CLU promoter that regulate IR inducibility of the gene have been difficult to examine since endogenous sCLU, as well as exogenous CLU promoter-reporter gene activity, are induced by various transfection methods (data not shown). We are currently working on experimental gene

delivery techniques to solve these problems and elucidate the factors required for CLU promoter expression after IR.

The signaling pathway resulting in sCLU induction after IR exposure is also unknown. Our laboratory identified CLU as a Ku70 binding protein using yeast-two-hybrid analyses (21). We discovered that a novel nuclear form of the protein, nuclear CLU (nCLU) bound Ku70 in co-immunoprecipitation experiments, but that sCLU did not associate with Ku70. Through our screen of cytotoxic agents, we found that DNA damage was not required for sCLU induction. This was best demonstrated by the induction of sCLU after thapsigargin treatment (Table 1). Thapsigargin (TG) is an inhibitor of the SERCA pump in the ER, and is required for calcium homeostasis. Treatment of MCF-7 cells with TG resulted in a transient release of intracellular calcium (56). Treatment of MCF-7 cells with TG alone resulted in an induction of sCLU mRNA and protein, suggesting that calcium changes may be an upstream signaling event mediating sCLU induction. It is possible that calcium, as a signaling molecule, may be a triggering event common to all the agents in Table 1 that elicit sCLU induction responses. The mechanism of this signaling pathway is currently being investigated in our laboratory.

The mechanism of sCLU repression by p53 also remains to be elucidated. There are several proposed models of p53 transcriptional repression. In the first model, p53 binds to its putative DNA binding sequence and sterically inhibits the binding of transcription factors required for induction. This model was proposed to account for repression of Bcl-2 (57), α-fetoprotein (58) and HBV (59) genes by p53. In the second model, p53 binds and sequesters transcription factors required for upregulation. For example, p53 can directly bind several transcription factors including Sp-1 (60,61), AP-1 (62), NF-Y (63,64), Brn-3a (57) and C/EBPβ (65), that may be responsible for upregulated CLU promoter activity after IR. Additionally, it

was shown that p53 can bind the TATA binding protein (TBP) *in vitro* and inhibit transcription by disrupting formation of the TFIID complex (66). Alternatively, Johnson *et al.* have proposed a novel putative DNA binding sequence for p53 that is strictly involved in transcriptional repression (67).

Our data strongly suggest that the CLU gene is negatively regulated by p53. The cell models used in this study will allow us to further investigate the mechanism(s) of p53 repression of sCLU, as well as the signaling pathways required for sCLU induction after IR exposure. Understanding the cellular responses to ionizing radiation exposure, in normal and tumor tissue, is vital for improving the efficacy of radio-therapy in the clinic. Additionally, elucidating mechanisms underlying sCLU induction may allow us to use this protein to improve the effectiveness of other chemotherapeutic agents. The data presented in this paper provide a first examination of how a cell may regulate the clusterin molecular switch, turning on the cytoprotective sCLU gene at low doses of IR (0.02 - 0.1 Gy) where p53 responses are not growth suppressive. At the same time, p53 responses after high doses of IR (≥ 1.0 Gy) appear to be responsible for shutting down this cytoprotective protein to allow for cell cycle checkpoint responses and for cell death in severely damaged cells. At higher doses of IR, p53 acts to suppress cytoprotective cell functions (e.g., sCLU expression) and at the same time mediate cell death, possibly via bax expression (32). Understanding these regulatory events after IR should allow elucidation of ways to modulate death responses in tumor cells and survival responses in normal cells.

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FIGURE LEGENDS

Figure 1. sCLU is transcriptionally upregulated after IR exposure in MCF-7:WS8 breast cancer cells. CLU mRNA levels were monitored in asynchronous MCF-7 cells after 10 Gy IR exposure by northern blot analyses and luciferase assays. In *A,* Log phase growing MCF-7 cells were irradiated with 10 Gy and 10 μg of total RNA was analyzed by northern blot analyses as described in *Experimental Procedures*, In *B,* MCF-7 cells were irradiated with various doses of IR and total RNA was harvested 72 hours after exposure. Total RNA (10 μg) was used for northern blot analyses. In *C,* Time-course of sCLU induction after 10 Gy IR exposure was analyzed by luciferase assays in MCF-7 cells stably transfected with 1403 base pairs of the CLU promoter (i.e., MCF-7 1403 cells) using the Luciferase Assay System (Promega). In *D*, An IR dose-response was performed on the MCF-7 1403 cells 72 h after IR exposure. Each dose/time point was performed in triplicate and a Student's T-Test was performed to determine statistical significance.

Figure 2. sCLU basal levels are elevated in MCF-7 cells that overexpress the HPV E6 protein and shows little induction after IR exposure. MCF-7:parental and E6D cells were exposed to 10 Gy and protein was harvested at various time points. In *A,* protein (100 μg) was loaded for each sample and separated by standard 10 % SDS-PAGE. Blots were probed for sCLU, p53 and Ku70 using western blot analyses as described in *Experimental Procedures.* Ku70 was used as a loading standard as described. In *B,* Total RNA (10 μg) was analyzed using standard northern blot techniques as described in Fig. 1. Shown are representative blots from experiments performed at least three times.

Figure 3. sCLU is induced in HCT116 p53 null cells, but not in p53$^{+/+}$ parental HCT116. Asynchronous HCT116 parental and p53 $^{-/-}$ cells were exposed to 10 Gy and protein was harvested at various time points. In *A,* Western blot analyses were performed as in Fig. 2. Blots were probed for sCLU, p53 and Ku70 by western blot analyses as described in *Experimental Procedures*. In *B,* Total RNA (10 μg) was analyzed using northern blot techniques as described in Fig. 1 and *Experimental Procedures*. Shown are representative blots from experiments performed at least three times.

Figure 4. sCLU is not induced in HCT116 p21 $^{-/-}$ cells. HCT116 p21 $^{-/-}$ cells were exposed to 10 Gy and protein was harvested at various post-treatment times as described. In *A,* Western blot analyses were performed as in Fig. 2. Blots were probed for sCLU, p53 and Ku70 by western blot analyses as described in *Experimental Procedures*. In *B,* Total RNA (20 μg) was analyzed using standard northern blot techniques as described in Fig. 1. Shown are representative blots from experiments performed at least three times.

Figure 5. sCLU is not cell cycle regulated. HCT116 parental, p53 $^{-/-}$ and p21 $^{-/-}$ cells were synchronized by serum starvation and confluence-arrest. Synchronized cells were released by low density seeding in 10% FCS-DMEM medium and cells were either allowed to proceed through the cell cycle after mock-irradiation (Fig. 5A, C, E), or irradiated with 10 Gy (Fig. 5B, D, F) at 10 h after release. Cells were allowed to proceed through G_1 (●), S (▼) and G_2/M (○) phases of the cell cycle. Protein was harvested for flow cytometric or western blot analyses at the indicated time points as described previously in *Experimental Procedures*. Western blots

were probed for sCLU, p53 or Ku70 expression as described in *Experimental Procedures*. Shown are HCT116 $p53^{+/+}$ parental (A, B), HCT116 $p53^{-/-}$ (C, D) and HCT116 $p21^{-/-}$ (E, F) cells. Shown are representative blots and cell cycle distribution changes for experiments performed at least three times.

Figure 6. Proposed model of the regulatory elements controlling sCLU expression. We propose that there are two opposing forces that control the expression of sCLU. In order to have net sCLU induction after IR, activating transcription factors must overcome the p53 transcriptional repression.

Table 1: Induction of sCLU protein expression in MCF-7 cells[1] by various cytotoxic agents.

Agent	Dose Range for Induction[2]
DNA Damaging Agents	
Ionizing Radiation (IR)	0.02 - 10 Gy
Ultraviolet Radiation (UV)	12 J/m^2
Photodynamic Therapy (PDT)[3]	200 nM PC-4/200 mJ/cm^2
Topotecan	50 nM
Camptothecin	100 nM
VP-16	15 uM
Non-DNA Damaging Agents	
Colcemid	70 ng/ml
Nocodazole	150 ng/ml
Taxol	1 - 50 nM
Taxotere	1 - 10 nM
Mimosine	0.5 mM
TPA	100 nM
Thapsigargin	10 - 500 nM

[1]Log phase asynchronous MCF-7 cells were seeded at approximately $5X10^5$ cells per 10 cm plate.

[2] Topotecan, camptothecin and TPA were continuous treatments. Cells were treated with colcemid, nocodazole and mimosine for 24 h, washed with PBS and replated into fresh media. Cells were treated with taxol and taxotere for 4 h, washed with PBS and replated into fresh media. Cells were treated with thapsigargin and VP-16 for 1 h. Protein was harvested at least 48 hours after drug addition/ irradiation. Induction of sCLU was scored as positive if ≥ 2-fold increases in sCLU levels were noted between 48-72 h post-IR treatment.

[3]Photosensitizing drug used was Phthalocyanine 4 (PC-4). Induction of sCLU protein was only seen after addition of drug and light exposure. No induction was observed with light alone or PC-4 alone.

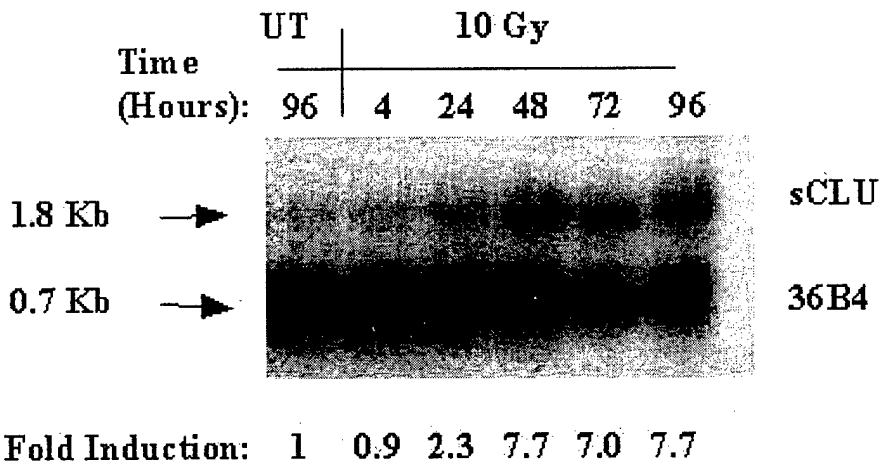
Table 2: Effect of p53 status on sCLU basal and IR inducible expression in a limited number of breast cancer cells.

		sCLU expression		
Cell Line	**p53 Status**	**Basal[1]**	**IR Inducibility[2]**	**RNA[3]**
MCF-7:parental	wild-type (wt)	low	yes	+
MCF-7:E6D	wt (no expression)	high	yes	+
ZR-75-1	wt	low	no	+
T47-D	mutant (194)	high	no	+
BT474	mutant (275)	high	no	+
MDA-MB-231	mutant (280)	not detected	not detected	+
MDA-MB-468	mutant (273)	high	no	+
HCT116 parental	wt	low	minimal	+
HCT116 p21 $^{-/-}$	wt	low	minimal	+
HCT116 p53 $^{-/-}$	null	low	yes	+

[1]Basal levels determined as compared to log-phase growing untreated MCF-7 parental cells.
[2]Log-phase growing cells were treated with 10 Gy IR and protein was harvested 48 hours after exposure. MCF-7 parental cells were used as the standard for "high" IR inducibility.
[3]RNA status was determined by RT-PCR using primers designed to full length CLU.

Figure 1

A)


UT
10 Gy
Time
(Hours):
96
4
24
48
72
96
1.8 Kb
0.7 Kb
sCLU
36B4
Fold Induction:
1
0.9
2.3
7.7
7.0
7.7

B)

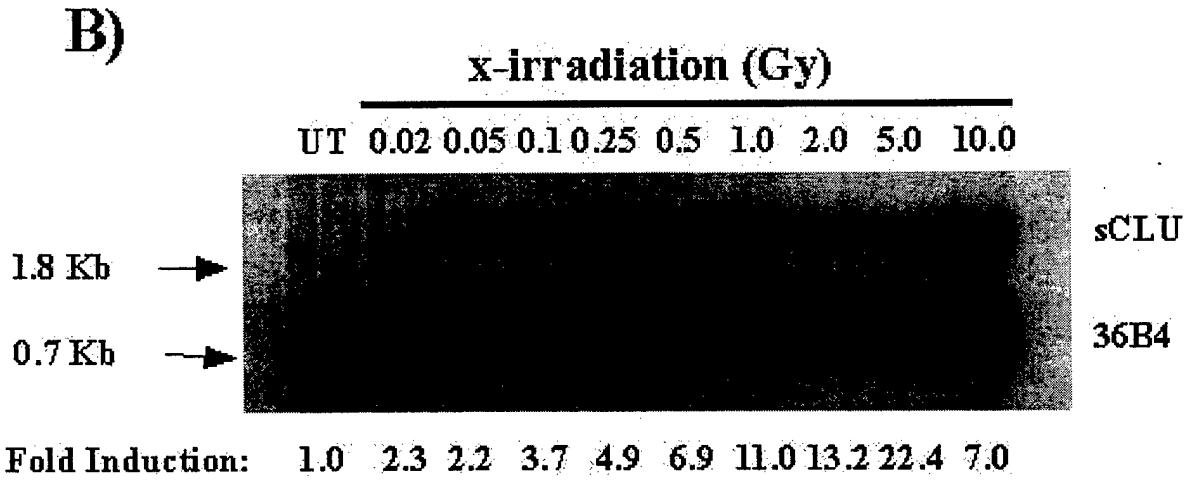
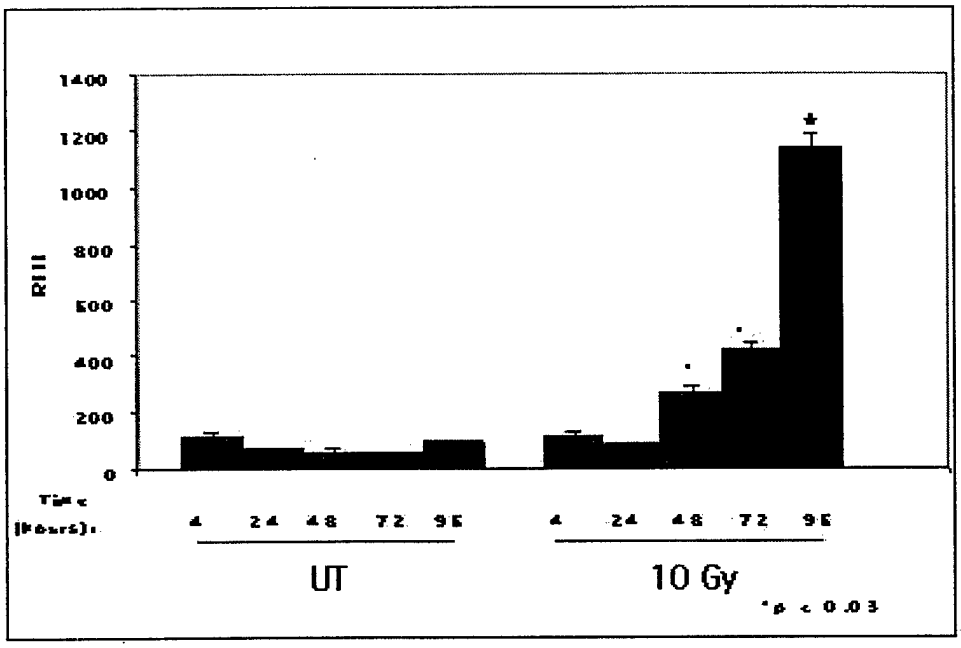

x-irradiation (Gy)
UT
0.02
0.05
0.1
0.25
0.5
1.0
2.0
5.0
10.0
1.8 Kb
0.7 Kb
sCLU
36B4
Fold Induction:
1.0
2.3
2.2
3.7
4.9
6.9
11.0
13.2
22.4
7.0

Figure 1 (cont.)

C)



D)

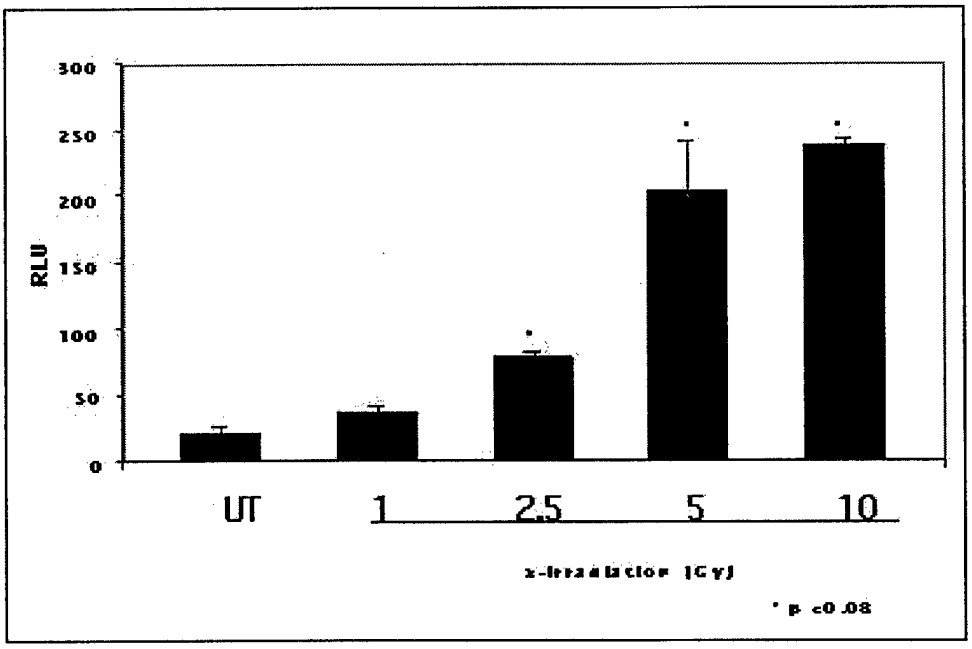
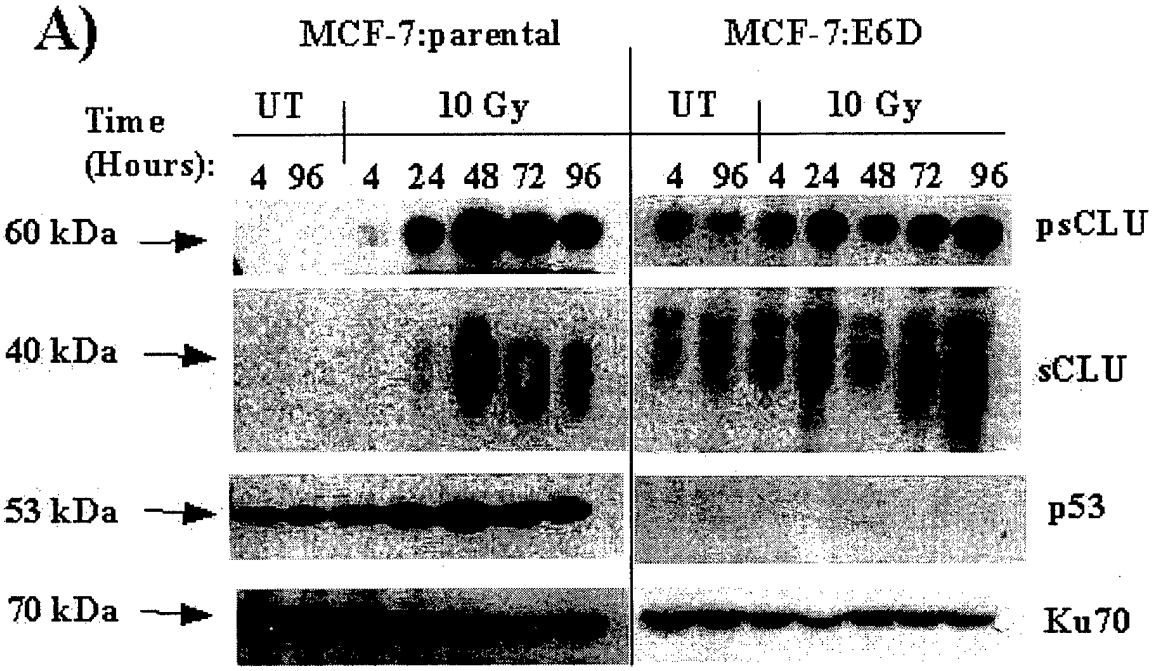
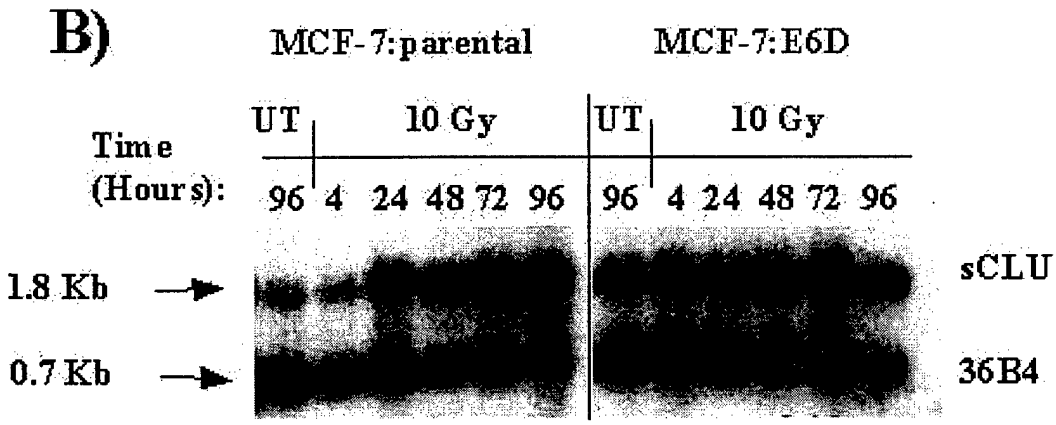


Figure 2

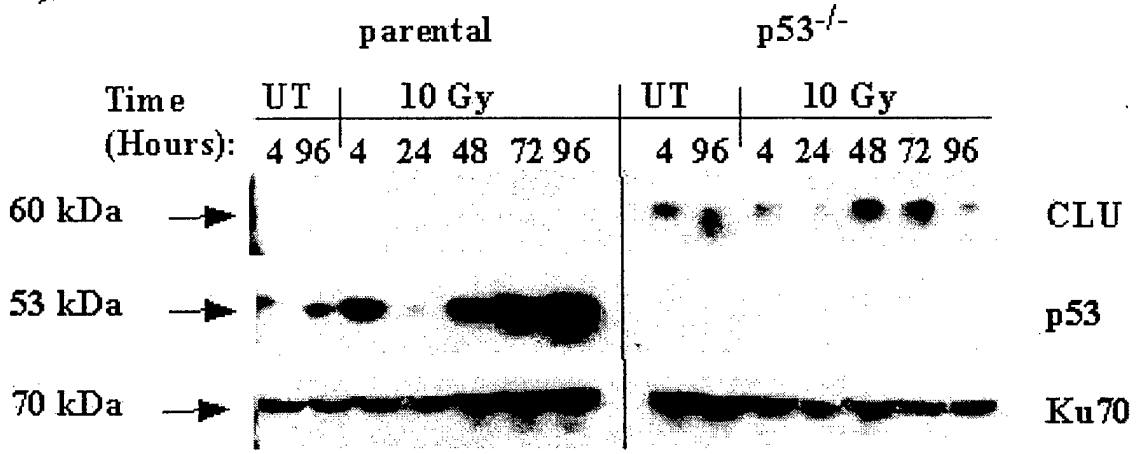




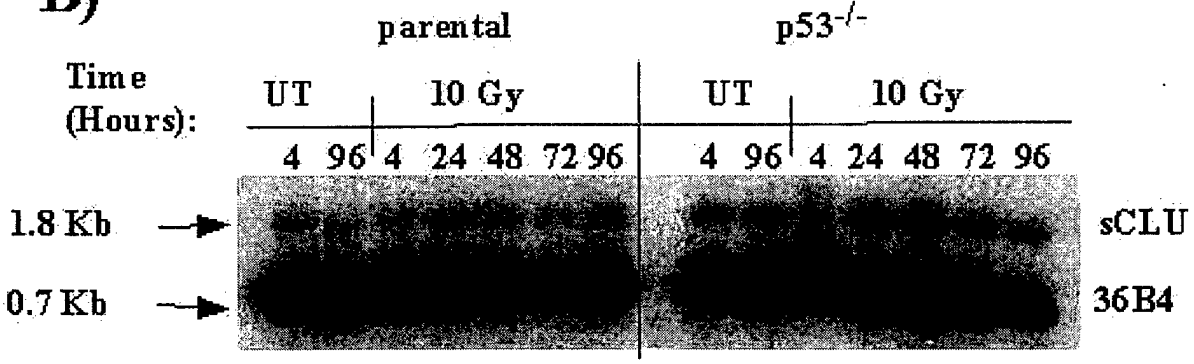
Fold Induction: 1.0 1.2 2.7 8.2 9.7 8.5 2.9 2.7 3.15.3 7.9 8.5

Figure 3

A)



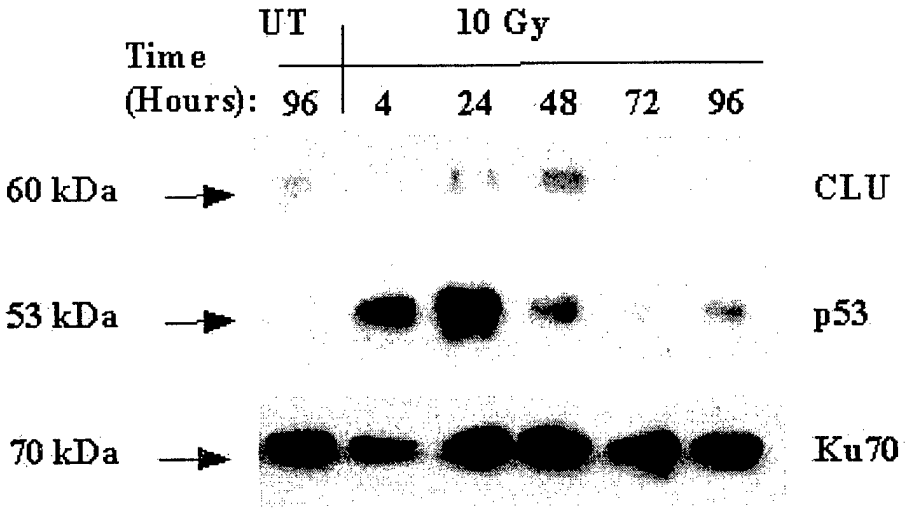
B)



Fold Induction: 1.0 0.4 1.0 1.0 1.4 1.3 1.3 1.1 1.0 2.3 1.5 1.7 1.9 2.3

Figure 4

A)


UT
10 Gy
Time
(Hours):
96
4
24
48
72
96
60 kDa
CLU
53 kDa
p53
70 kDa
Ku70

B)

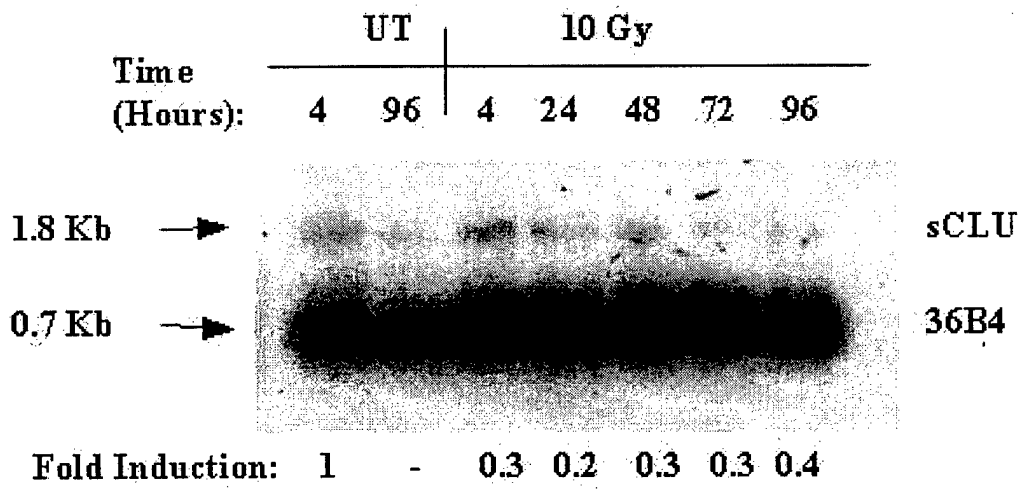

UT
10 Gy
Time
(Hours):
4
96
4
24
48
72
96
1.8 Kb
sCLU
0.7 Kb
36B4
Fold Induction:
1
-
0.3
0.2
0.3
0.3
0.4

Figure 5

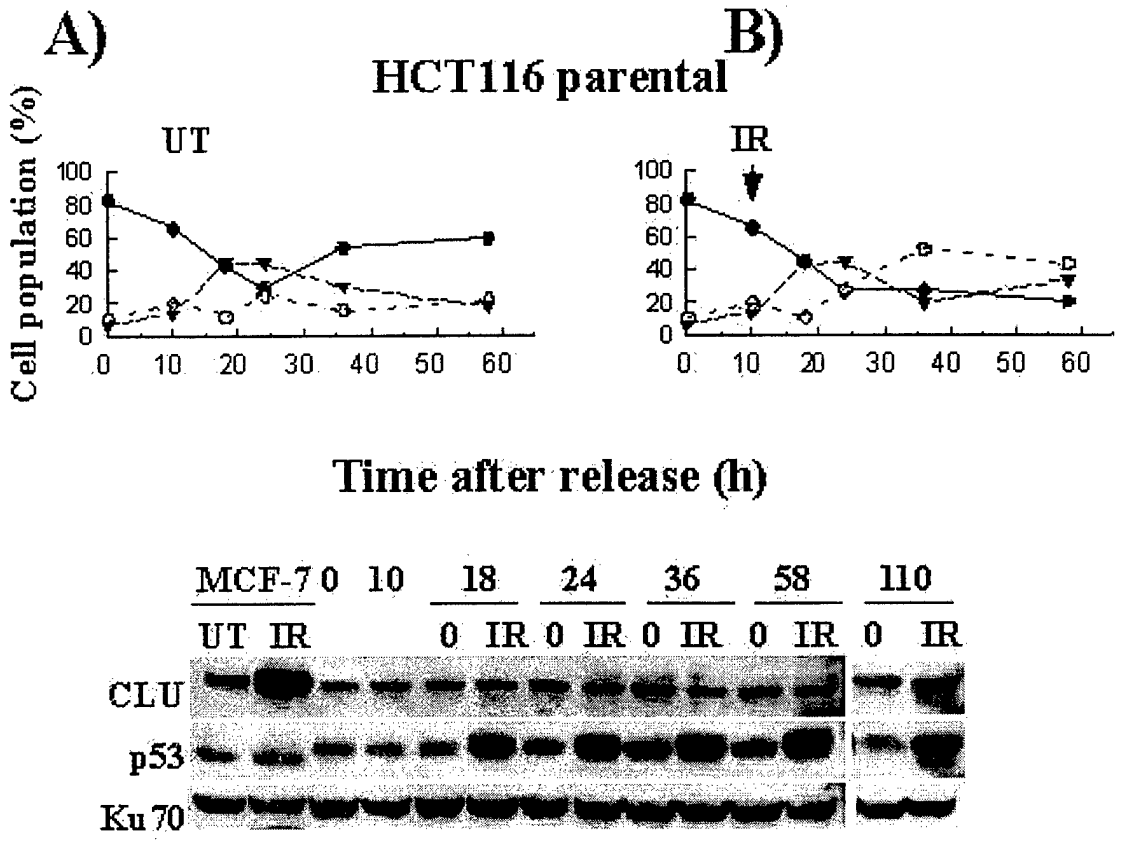

A)
B)
HCT116 parental
UT
IR
Cell population (%)
100
80
60
40
20
0
0 10 20 30 40 50 60
Time after release (h)
MCF-7 0 10 18 24 36 58 110
UT IR 0 IR 0 IR 0 IR 0 IR 0 IR
CLU
p53
Ku 70

Figure 5 (cont.)

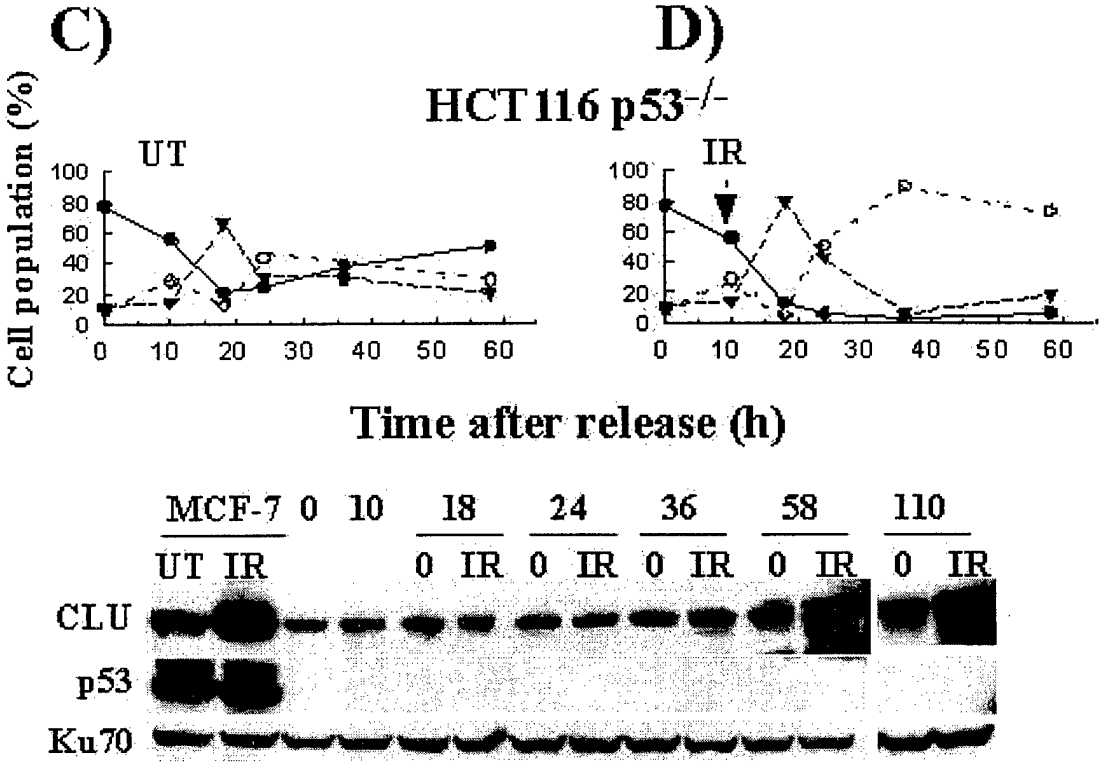


Figure 5 (cont.)

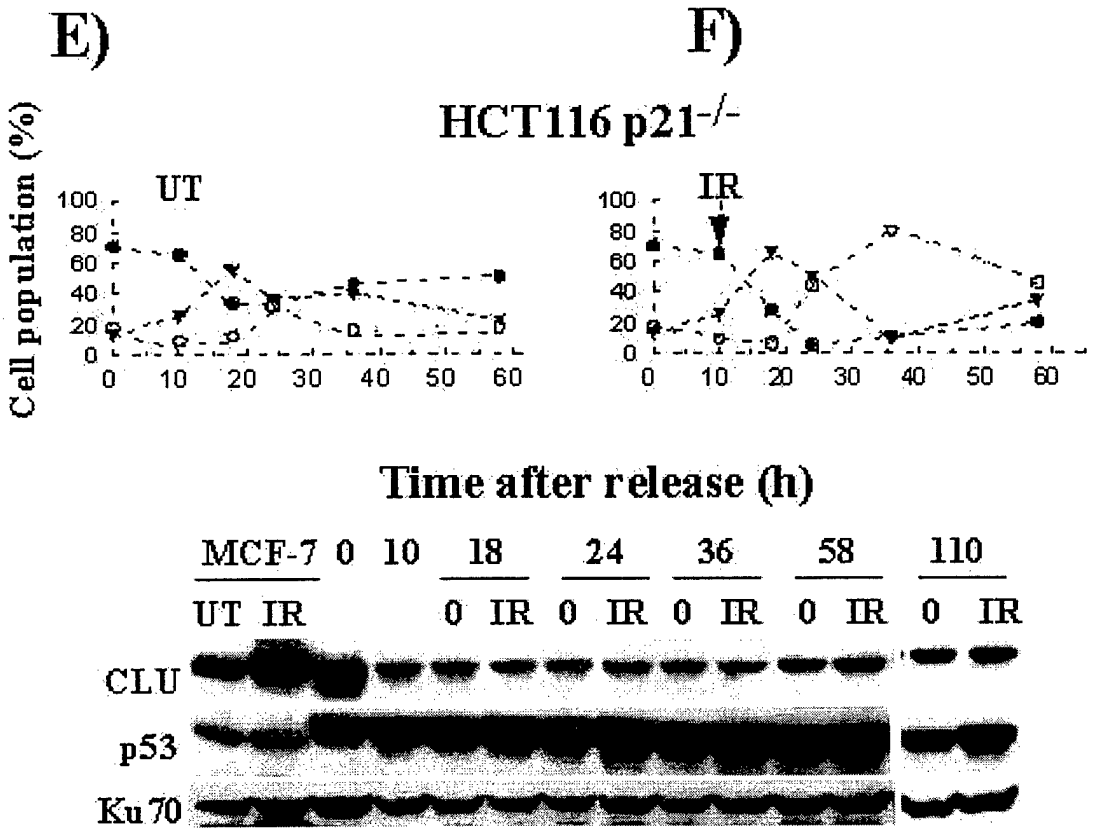
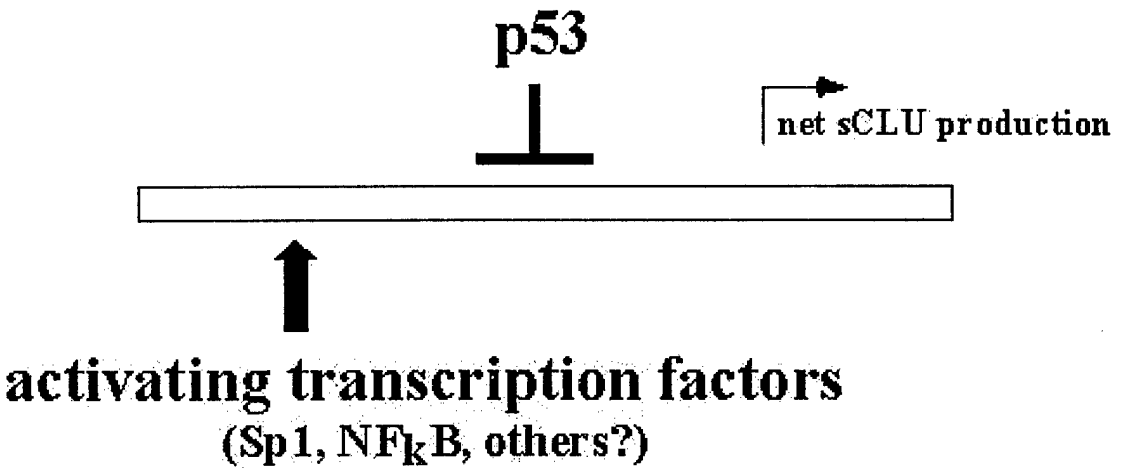


Figure 6


p53
net sCLU production
activating transcription factors
(Sp1, NFkB, others?)